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14. ABSTRACT
Our goal is to ultimately enable the development of novel therapeutic agents and strategies to ameliorate tissue injury in I/R related conditions. Preliminary findings suggested a role for Il-17 & IL-23 axis in ischemia reperfusion injury which we have continued to study. We have shown that by depleting IL-17 and IL-23 tissue injury is alleviated.

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Table of Contents

W81XWH-12-1-0526

Introduction

Tissue injury due to mesenteric ischemia/reperfusion is a common pathology that is frequently encountered in a variety of clinical and surgical conditions including shock, trauma, small bowel transplantation, sepsis, and cardiovascular interventions. Ischemia induces local cellular changes such as membrane perturbation, cytoskeletal disorganization, upregulation of adhesion molecules, neo-antigen expression and increased permeability. These alterations provoke endothelial dysfunction, intense inflammatory cascade, increased mucosal permeability and tissue necrosis during subsequent reperfusion (1-10). The susceptibility of the ischemic tissue to the ensuing catastrophic events caused during the re-establishment of blood, leads to a phenomenon known as ischemia/reperfusion (I/R) injury. The restored blood flow also causes systemic inflammation affecting remote organs resulting in systemic inflammatory response syndrome and multiple organ dysfunction syndromes that are associated with high rates of morbidity and mortality (10).

Several molecules and cells have been implicated in I/R injury, such as reactive oxygen species (ROSs), cytokines and chemokines (11), adhesion molecules (12,13), complement (14,15), natural antibodies (16-18), B cells (19), T cells (20, 21), neutrophils (22), endothelial cells and platelets (23). Deposition of natural antibodies, subsequent complement activation and neutrophil infiltration represent the initiating events in this process (9). Recently, the role of different T cell subsets (Tregs, Th17) and B cells in I/R injury has been considered as well (24, 25). In addition, there are increasing reports on the role of gut microbiota in acute intestinal inflammation and injury (26). Data from the ongoing studies clearly show that I/R injury is a very complex process that literally recruits all the components of the immune response. To date there is no specific therapy that has yet gained widespread clinical use for the prevention and treatment of ischemic related conditions.

Others and we have shown that natural antibodies are central in the pathogenesis of mesenteric I/R injury and initiate I/R-induced tissue injury (14, 16, 17). The observations led to the identification of neo-antigens such as annexin IV and β-actin on the surface of the ischemic cells to which natural IgM bind and subsequently activate complement (27, 28). Deficiency of complement factors has been shown to be protective against I/R injury (14), and inhibition of the complement pathway through various interventions have been reported to attenuate I/R-induced damage (29). These findings clearly indicate the phlogistic behavior of these key molecules in I/R injury and their potential to be targets for therapeutic intervention. We are continuing our efforts to further understand and reveal the underlying mechanisms associated with different aspects of I/R pathology and counter the processes using various pharmacological agents and therapeutic strategies.

Body

Goal 3.2 Determine whether anti-IL-17 antibody treatment prevents IR and HR injury.

We have determined that IL-17 is important in IR injury. We have shown decreased intestinal damage after IR injury in IL-17 KO mice. Furthermore we showed that the attenuation in intestinal injury after IR was due to the lack of IL-17 in the KO mice and not some other aberration that occurred during development of the KO mouse by showing that WT mice when treated with a neutralizing antibody to IL-17 also have decreased intestinal injury when compared to WT mice injected with isotype control antibody.

Ischemic injury induces the expression by damaged cells of antigens that are recognized by circulating natural antibodies, which fix and activate complement. This is supported by the observation that IgM, IgG and C3 are deposited in the small intestine during IRI, by the abrogation of IRI in the absence of complement and the ability of serum from WT and lupus-prone MRL.*lpr* mice to restore IRI in *Rag*-deficient IRI-resistant mice. We asked whether IL-17 precedes or follows the deposition of Ig and complement action in the expression of IRI injury. In considering that *Il17a$^{-/-}$* mice could lack natural antibodies in a manner similar to that observed in *Cr2*-deficient mice, we administered purified IgG from MRL.*lpr* mice 30 min prior to the initiation of IR. Injection of lupus mouse derived Ig into *Il17a$^{-/-}$* mice failed to restore IRI (Fig. 1). The same batch of IgG restored IRI in *Rag2$^{-/-}$* mice in a manner similar to that shown before (data not shown). These results suggest that IL-7A is downstream of antibody- and complement-mediated triggering of IRI.

**Figure 1. IL-17A is downstream from the early events of IRI.**

Failure of IgG from B6MRL.*lpr* mice to restore IRI in *Il17a$^{-/-}$* mice. **A.** H&E Stained sections **B.** Chiu score.
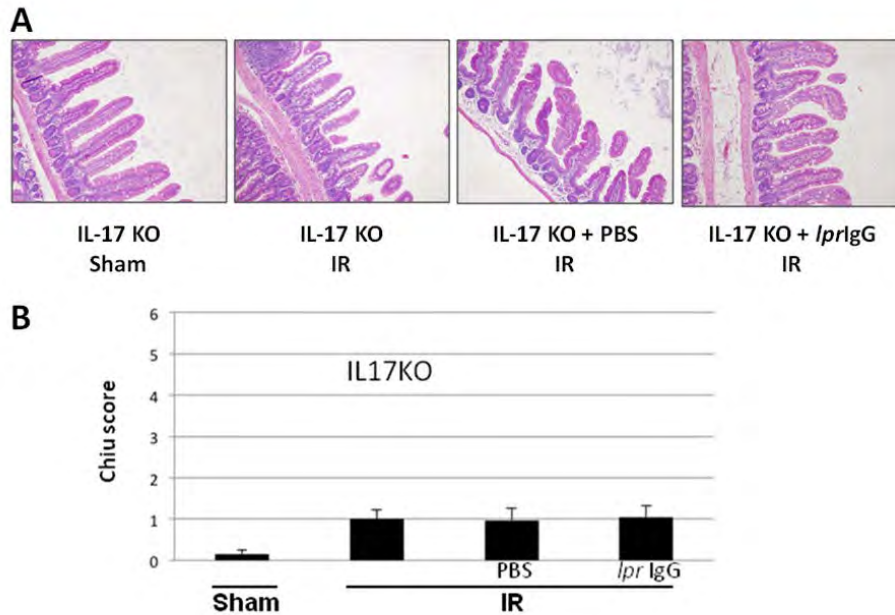
3.4 Use Rag deficient mice (lack T and B lymphocytes) which do not suffer IR injury, to determine the relative contribution of Th 17 cells in the expression of injury

Our data suggests that IL-17A is important for small intestinal IRI, and that Paneth cells store IL-17A and are the major source of IL-17A in IRI. This prompted us to analyze the potential cellular source of IL-17A important for small intestinal IRI. We first tested the hypothesis that ILCs, which rapidly release IL-17A in response to epithelial injury, are the relevant source of IL-17A in small intestinal IRI. To address this question we took advantage of the $Rag2^{-/-}\gamma c^{-/-}$ mouse, which lacks ILCs because it is deficient in IL-7 signaling, which requires the γc containing IL-7R. Since $Rag2^{-/-}\gamma c^{-/-}$ mice, like $Rag2^{-/-}$ mice, lack immunoglobulins, we administered IgG from MRL.*lpr* mice to $Rag2^{-/-}\gamma c^{-/-}$ mice prior to subjecting them to IR. Infusion of lupus-derived Ig reconstituted IRI in $Rag2^{-/-}\gamma c^{-/-}$ mice even better that it did in $Rag2^{-/-}$ mice (Fig 2. A,B). These results indicate that ILCs are not the relevant source of IL-17A in small intestinal IRI and suggest that ILCs may in fact protect against small intestinal IRI.

Figure 2. ILCs are not the source of IL-17A in IRI. A-B. Representative H&E stained intestinal sections **A.** Chiu scores **B.** in $Rag2^{-/-}\gamma c^{-/-}$ mice reconstituted with IgG from MRL.*lpr* mice.

A

| | WT | Rag2⁻/⁻ | Rag2⁻/⁻γc⁻/⁻ |

B
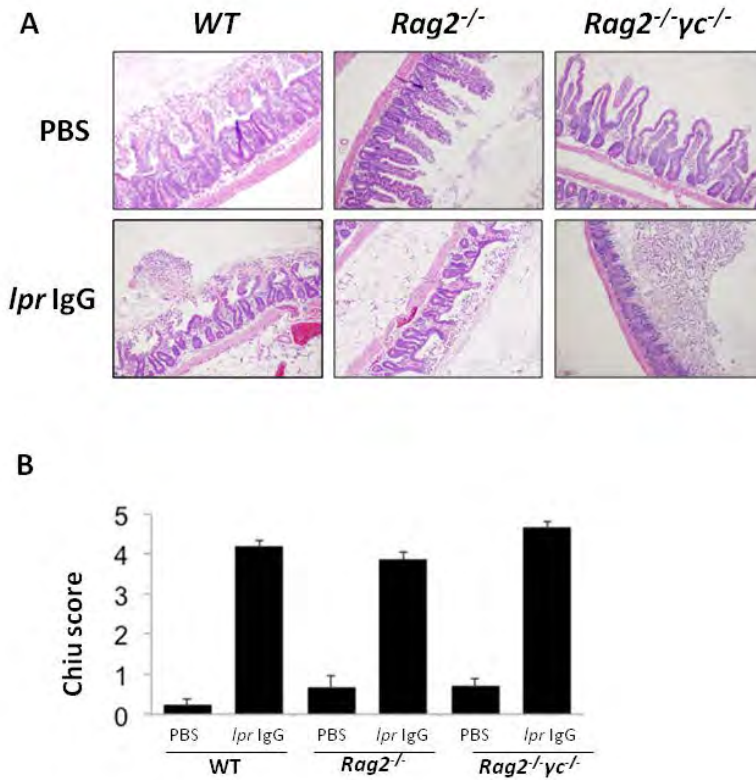
There are some published reports that suggest that Paneth cells produce *Il-17a*. However the published gene expression analyses do not show that Paneth cells express *Il17a* mRNA. In view of the above negative result and to ascertain whether a hematopoietic cell is the source of IL-17A in small intestinal IRI, we constructed bone marrow (BM) radiation chimeras. To determine the contribution of IL-17A derived from hematopoietic cells in small intestinal IRI, we generated chimeras in which BM from CD45.2[+] WT and *Il17a*⁻/⁻ donors was used to reconstitute lethally irradiated CD45.1[+] WT recipients. To determine the contribution of IL-17A derived from non-hematopoietic cells BM from CD45.1[+] WT donors was used to reconstitute lethally irradiated CD45.2[+] WT or *Il17a*⁻/⁻ recipients. FACS analysis revealed that after 8 weeks >90% of the blood cells in the BM chimeras are donor-derived. We are currently reconstituting the normal intestinal microbiota by cobedding with non-irradiated mice. These mice will be subject to intestinal IRI to determine whether or not the cellular source of IL-17a is of hematopoietic origin. If it is potential cellular sources of IL-17 would me neutrophils, mast cells, dendritic cells and macrophages. We will then investigate each of these cell types as a cellular source of IL-17A in IRI using the appropriate knockout mouse.

Local infiltration with neutrophils has been well documented in IRI, based on histologic examination of intestinal tissue sections and considered responsible for the development of

injury. Because the intestinal lesions in IRI can be patchy, and in order to measure neutrophil infiltration quantitatively, we determined the total number of neutrophils in the LP of intestine of mice subjected to IR. Cell suspensions were prepared from the LP of the entire small intestine and analyzed by flow cytometry analysis for CD45 expression. The percentage of neutrophils among the gated CD45$^+$ cells was determined using CD11b and Gr1 as markers. IRI resulted in an increase in the percentage and number of CD45$^+$ cells in the LP and in a time-dependent increase in the percentage of neutrophils in the CD45$^+$ fraction of cells (Fig. 3). These results demonstrate that IRI causes a time dependent accumulation of neutrophils in the LP of the small intestine.

Figure 3. Increased numbers of tissue infiltrating neutrophils in the small intestine following IR. A-C. Representative FACS analysis and percentage of CD11b$^+$Gr1$^+$ cells in the gated CD45$^+$ population of cells from the LP of WT mice subjected IR and sham treated controls. Bars represent SD. n=4 per group. * p<0.05.
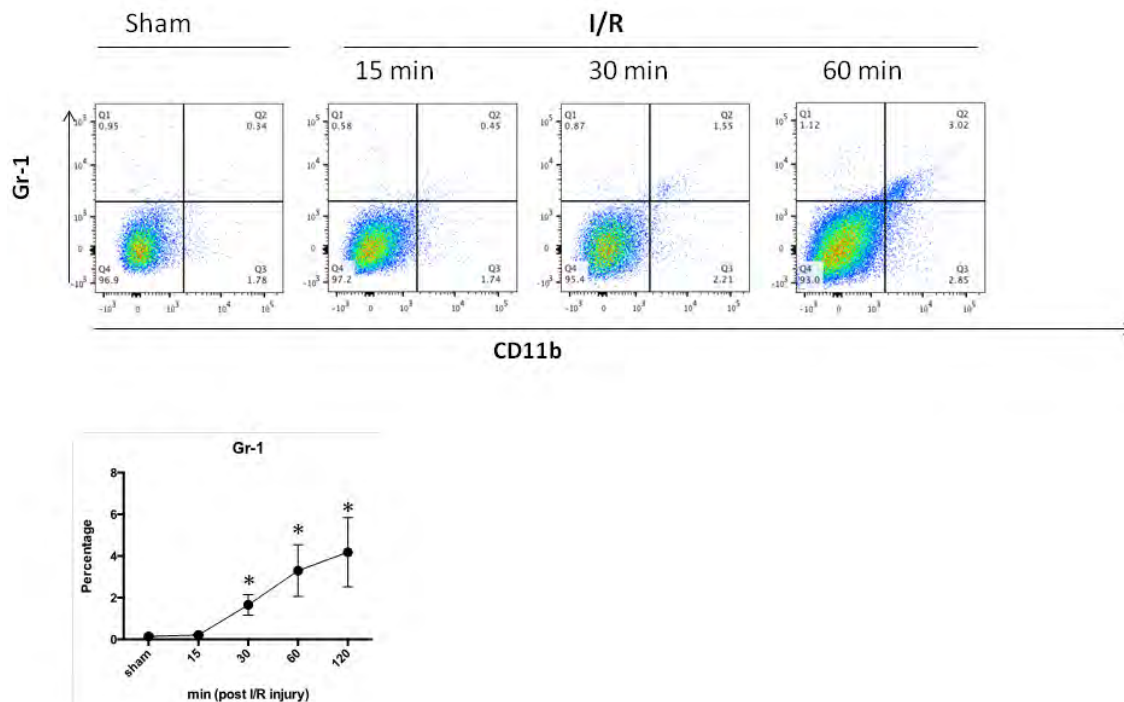
Protective role of R-spondins in I/R injury. A number of pharmacological agents and therapeutic strategies to attenuate the immune response triggered by intestinal I/R have been proposed, including the use of anti-oxidants agents, leukocyte and lymphocyte depletion and inhibition of complement activation. Despite significant advances in this field, no agent or

therapy has yet gained widespread clinical use for the prevention and treatment of ischemic related conditions. Considering the growing number of studies that implicate the beneficial role of R-spondins, we asked if these proteins could also prevent inflammation-associated tissue damage in ischemic disorders. Previously, our findings reveal that R-spo3 protects tissues against mesenteric I/R by tightening endothelial cell junctions and improving vascular integrity, which consequently dampens vascular leakage and the commencement of inflammatory events triggered during reperfusion.

Altered endothelial barrier function is a hallmark of numerous autoimmune and inflammatory conditions including I/R. Several studies have implied that ischemia/hypoxia perturbs actin dynamics in vascular endothelium, which affects junctional integrity and promotes increased vascular permeability. Our preliminary in vitro data present evidence that Rspo3 can prevent endothelial leakage, tighten endothelial cell junctions, preserve localization of VE-cadherin at the cell periphery and reduced stress fiber formation (F-actin levels) under inflammatory conditions. These findings dictate further studies to delineate the underlying molecular mechanisms of R-spo3 signaling that are responsible to stabilize cell junctions and preserve the endothelial barrier function under hypoxic stress.
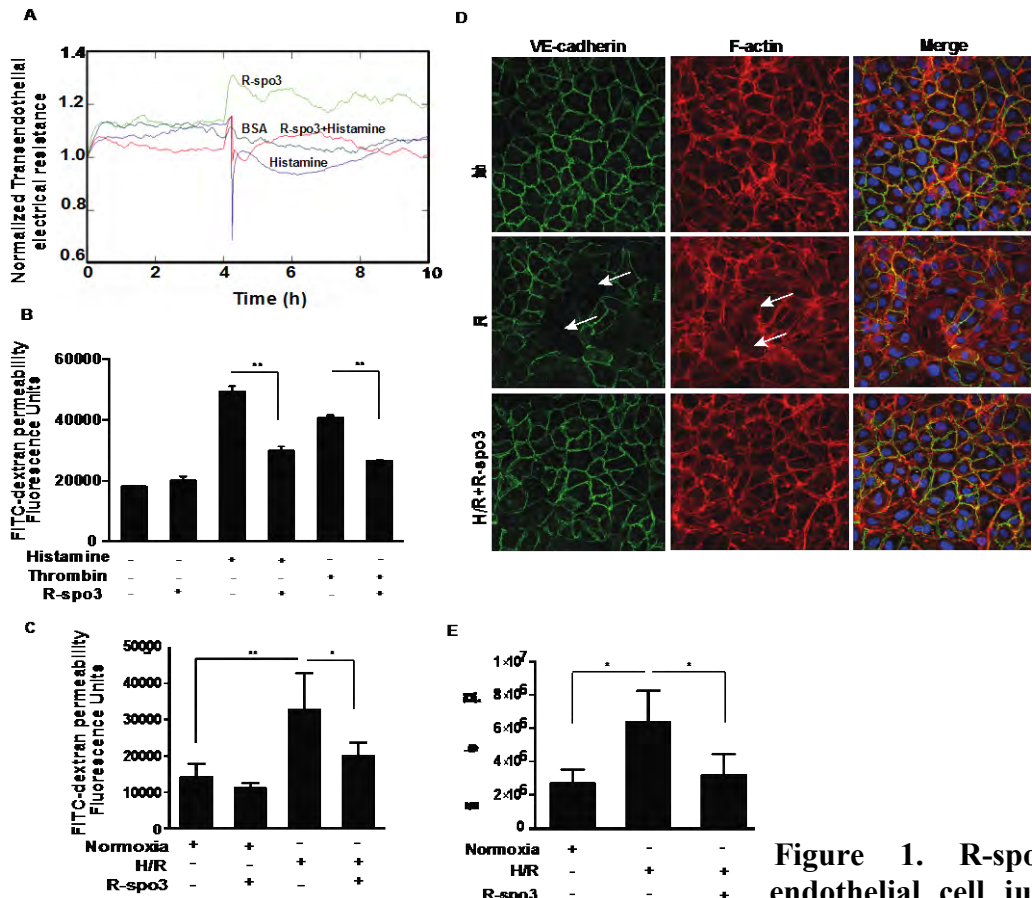

Figure 1. R-spo3 tightens endothelial cell junctions and preserves barrier function. (A) Effect of R-spo3 on endothelial junctional integrity, as measured by transendothelial electrical resistance (TEER) in HUVECs exposed to different conditions such as BSA, histamine, R-spo3 and Rspo3+histamine. (B) R-spo3 pretreatment

decreased histamine and thrombin-induced endothelial permeability in HUVECs measured using FITC-dextran passage (n= 4, **$p<0.01$). (C) R-spo3 pretreatment decreased H/R-induced endothelial leakage in HUVECs measured using FITC-dextran passage (n=5, *$p<0.05$, **$p<0.01$). (D) Effect of R-spo3 on localization of VE-cadherin (adherens junction marker) and F-actin was evaluated by immunofluorescence and phalloidin staining in HUVECs subjected to H/R. H/R resulted in the loss of the VE-cadherin at the cell junctions and increased the number of stress fibers (arrows), while R-spo3 preserved VE-cadherin and F-actin at the cell periphery and decreases the formation of stress fibers in cells subjected to H/R. One representative image of three experiments is shown. (E) Quantification of F-actin content in HUVECs under normoxic, H/R and H/R+R-spo3 conditions. Cumulative data from three independent experiments *$p<0.05$.
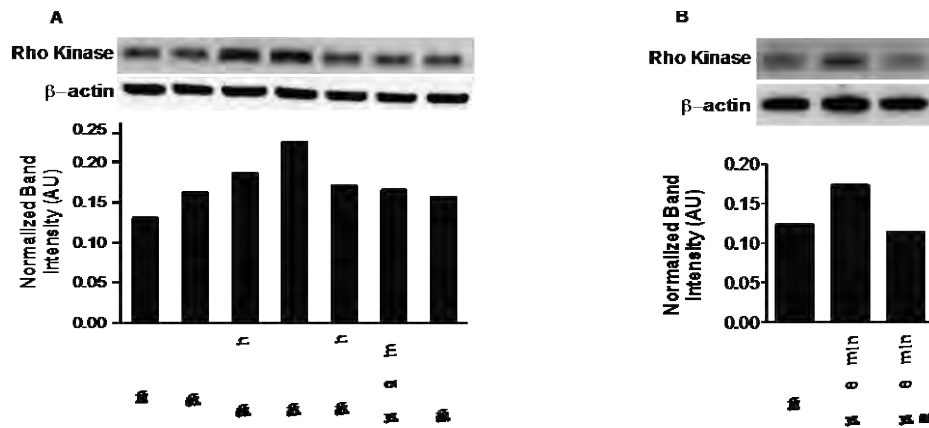

Figure 2: Hypoxia-induced changes in Rho kinase expression in HUVECs. HUVECs seeded on 35-mm dishes (5 X 10^5 cells per dish) were grown under normoxic conditions for 24 h. Prior to hypoxic conditions, the culture media was changed to the deoxygenated culture medium and cells were placed into anaerobic chamber. After subjecting cells to hypoxia for the indicated time periods- 5, 15, 30, 60, 120 and 240 min, cells were lysed and used for immunoblotting with anti-Rho kinase and anti-ß actin antibodies. Data are representative of three independent experiments. Data were quantified by densitometric analysis and the Rho kinase level is presented relative to ß actin expression. (B) R-spo3 suppresses hypoxia-enhanced expression of Rho kinase in HUVECs. HUVECs grown in normoxic conditions were subjected to hypoxia in the same manner as in A. Cells were lysed and used for immunoblotting with anti-Rho kinase and anti-ß actin antibodies. Data are representative of three independent experiments. Data were quantified by densitometric analysis and the Rho kinase level is presented relative to ß actin expression.
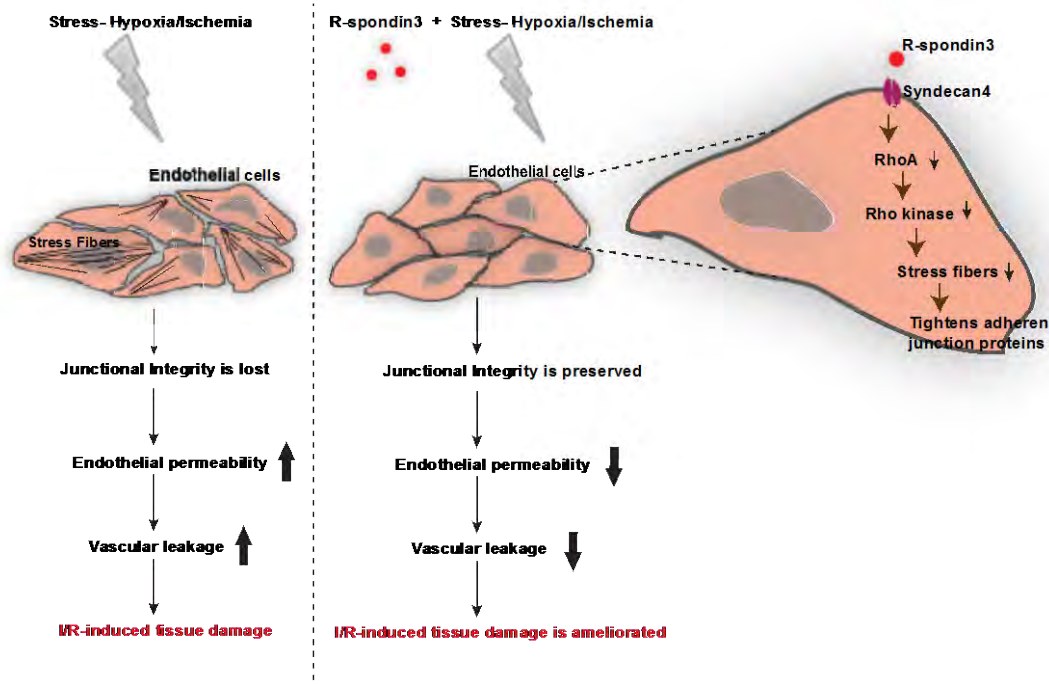
Figure 3: Proposed schema illustrates the protective role of R-spondin3 in endothelial cells under hypoxic stress, which is translatable to I/R conditions.

Key Research Accomplishments

IL17 and IL23 partially mediate tissue damage in IRI.

R-spondin3 improves endothelial barrier integrity by tightening intercellular junctions possibly through Rho GTPase signaling.

Reportable Outcomes

Poster presentation- American Association of Immunologists Annual Meeting: IL-17a is Important for Ischemia-Reperfusion Injury to the Small Intestine. Pittsburg, PA 2014.

Poster Presentation- American Academy of Pediatrics National Conference and Exhibition. The Role of IL-17a in Ischemia-Reperfusion Injury to the Small Intestine. Orlando, FL 2013.

Poster Presentation- Keystone Symposia on Hypoxic signaling: Role of R-spondin3 in Ischemia/Reperfusion Injury. Breckenridge, CO 2014.

Conclusions

Thus, the above mentioned studies qualifies the goal of our laboratory to ultimately enable the development of novel therapeutic agents and strategies to ameliorate local as well as remote tissue injury in I/R related conditions.

R-spondin3 protects intestine from mesenteric ischemia/reperfusion induced tissue damage by tightening endothelial cell junctions and preventing vascular leakage. Thus, our findings unravel the therapeutic role of R-spondin 3 in protecting tissues from damage by preventing endothelial barrier dysfunction and dampening I/R enhanced local inflammatory response. We are now exploring in depth the regulatory mechanisms by which R-spo3 plays its protective role in tissue injury for therapeutic purposes. This study aims to support the idea of testing the therapeutic potential of physiologically relevant biological molecules in I/R related conditions encountered in clinical and surgical settings.

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